STUDIES ON THE MECHANISM OF IMMUNITY IN TUBERCULOSIS

The Rôle of Extracellular Factors and Local Immunity in the Fixation and Inhibition of Growth of Tubercle Bacilli

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Plates 32 to 34

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The investigations of Rich (1) and his associates have cast profound doubt on the rôle of allergy in immunity to tuberculosis. Their studies intimate that both the fixation of tubercle bacilli at the site of reinfection and the inhibition of their growth may take place in the absence of exaggerated inflammation. Many studies (2) since then have indicated that there is no strict parallel between allergy and immunity. If the fate of the bacilli of reinfection is determined it has been found (3) that their destruction and the inhibition of their growth on intravenous inoculation is most marked in the presence of considerable residual primary lesions and is associated with scant inflammatory response at the site of their focalization. With slight remaining primary tuberculosis the bacilli of reinfection are less effectively inhibited, yet the acute inflammation at the site of localization of the microorganism is much more intense. On introducing melted agar impregnated with tubercle bacilli and trypan blue subcutaneously into normal and highly immunized rabbits (4), it was found that with large doses, the early rush of lymph from the focus of reinfection is so intense that both the bacilli and the trypan blue are swept over to the draining lymph nodes much more rapidly in the sensitized than in the normal animal. However, one cannot conclude from this that in man also the exaggerated inflammation aids rather than hinders the dissemination of the bacilli of reinfection. Rabbits become only moderately sensitized to the tubercle bacillus

as compared to the exquisite sensitivity that man acquires as a result of a tuberculous infection. It has been shown by Menkin (5) that the fixing capacity of an inflammation is proportional to the injury exerted by the inflammatory agent on the tissues. Therefore the study with a local agar focus was repeated in the guinea pig, the acquired allergic sensitivity of which is more like that of man. It was the purpose of this endeavor to reexamine the immune reactions in the guinea pig and to determine if possible whether allergy plays any rôle in the fixation of the bacilli of reinfection. Incidentally observations have been made that bear on the problem of local immunity.

In the study with the agar focus where the body fluids readily penetrated the agar masses, but into which the cells entered slowly, it was shown that in the acellular agar islands of the normal animal the bacilli multiplied unhindered; in the immune animal, on the other hand, a marked inhibition of their growth was evident in these cell-free areas saturated, as they were, with the immune body fluids. In this paper, based on the use of another procedure, it is felt that more definite evidence of the rôle of humoral bacteriostatic factors in immunity to tuberculosis *in vivo* is presented.

Rôle of Extracellular Factors in the Fixation of Tubercle Bacilli of Reinfection

Methods and Materials

The use of the agar focus has been previously described (4). Guinea pigs were vaccinated with 2 mg. of BCG subcutaneously on the right side of the back near the shoulder. 38 days later, when they exhibited marked sensitivity to tuberculin, these, together with a group of normal guinea pigs, received subcutaneously over the left thigh 5 cc. of a mixture containing 6 per cent agar in saline solution adjusted to pH 7.4, virulent human type tubercle bacilli (P 15 B) and trypan blue. Another series of guinea pigs was given 1 mg. of R 1 tubercle bacilli, a strain of low virulence, intraperitoneally. 53 days later the same animals received 2 mg. of the same culture subcutaneously on the right side of the back near the shoulder. 62 days after this last treatment, the vaccinated, together with a group of normal animals, received 4 cc. of a mixture containing 4.5 per cent agar in saline solution, virulent bovine type tubercle bacilli (Ravenel strain), and trypan blue. In each series the number of bacilli present in the inoculum was determined by culturing unit weights of the agar suspension on Löewenstein's medium supplemented with bone marrow infusion as previously described (6).

At different intervals of time following inoculation a normal and a vaccinated animal were killed. The character of the local and metastatic lesions and their content of trypan blue was noted. The number of living tubercle bacilli present in a unit weight of tissue was determined by culture for the following structures of normal and vaccinated animals: the agar focus with its investing capsule, the superficial inguinal and deep iliac nodes draining this focus, the superficial inguinal nodes on the opposite side, *i.e.* the control inguinal node, and the spleen or liver. The number of colonies cultured from a given tissue was correlated

TABLE I

The Fate of Virulent Tubercle Bacilli and Trypan Blue in an Agar Focus and Their Dissemination in the Body of Normal and R 1 Vaccinated Guinea Pigs

Agar suspension	Time after inoculation	Agar focus				Draining inguinal nodes				Control inguinal nodes		Draining iliac nodes				Liver	
		Normal		Vaccinated		Normal		Vaccinated				Normal		Vaccinated			
		Colonies	Trypan blue	Colonies	Trypan blue	Colonies	Trypan blue	Colonies	Trypan blue	Normal	Vaccinated	Colonies	Trypan blue	Colonies	Trypan blue	Normal	Vaccinated
	days																
12,400	1	22,200	+++	5500	+++	10	+	0	±	−	0	20	±	0	tr.	0	0
	4	159,000	+±	4600	++±	1730	++	10	+	−	0	0	++±	*	+	0	0
	8	29,000	+±	48,300	+±	8000	+	†	†	−	0	18	+±	13	+±	40	4
	14	366,000	+	12,000	+±	38,600	+	0	tr.	690	0	94,500	+	36	tr.	2100	4
	28	16,600	−	730	−	10,600	−	600	−	−	0	74,900	−	110	−	18,700	7

The intensity of coloration of the agar focus and the draining lymph nodes is graded as follows: tr., trace of blue; ±, faintly blue; +, pale blue; ++, moderately blue; and +++, deep blue.

* Residual regressive tubercle from primary infection; 210 colonies were cultured therefrom.

† This lymph node was enmeshed in the agar focus; 720 colonies were cultured therefrom.

with the histological changes in the tissue immediately adjoining. The sections were stained with Masson's trichrome procedure as modified by Foote (7), by the Ziehl-Neelsen stain for tubercle bacilli and by Mallory's fibrin stain. The fate of the bacilli and the tissue response at the site of inoculation, as well as in the metastatic foci of the normal guinea pigs and those vaccinated with R 1, were compared to the host parasite interactions in the same foci of normal rabbits, and rabbits harboring a primary residual tuberculosis, and inoculated, like the guinea pigs, with comparable amounts of the same culture administered subcutaneously in melted agar and trypan blue. The detailed data for the rabbits have already been reported (4).

Fate of the Bacilli

Since the results obtained from the two guinea pig series were essentially the same, except that with BCG vaccination the immunity exhibited was much less pronounced than that shown by guinea pigs repeatedly treated with the more virulent R 1 culture, the protocols for the former are omitted. In Table I are presented the fate of the bacilli and trypan blue in the normal and R 1 vaccinated guinea pigs.

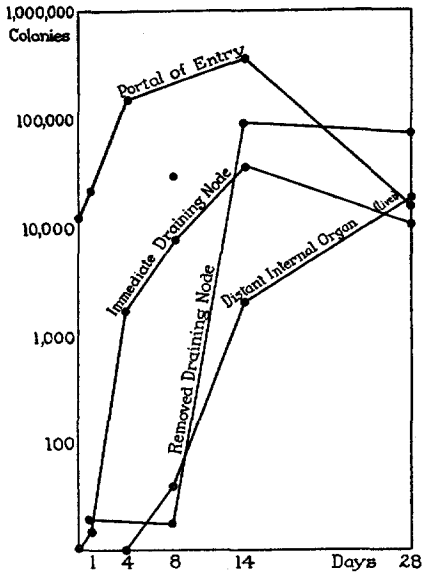
It will be noted that the inoculum contained a large number of tubercle bacilli, 12,400 colonies being isolated from 10 mg. of the agar suspension. The bacilli in the agar focus of the normal guinea pig multiply almost uninterruptedly up to the 2nd week. By the 4th week, however, there is a marked reduction in the number of tubercle bacilli. Essentially the same observations were made with the agar focus in rabbits (see Table III in previous study (4)). In these animals also the bacilli multiply up to the end of the 2nd week, and a marked reduction in their numbers is found in the 4th week. In the R 1 vaccinated guinea pigs, and in rabbits that harbored a primary infection, the multiplication of the bacilli in the agar focus is markedly inhibited from the beginning. The superficial inguinal node on the side opposite the agar focus, *i.e.* the control inguinal node, was sterile in every case, a fact suggesting that the bacilli cultured from the draining superficial nodes were derived from the bacilli that had invaded these structures from the focus of reinfection, except in the two instances noted in the table.

While the behavior of the bacilli of reinfection at the site of inoculation was the same in rabbits and guinea pigs, the dissemination of the bacilli of reinfection to the draining lymph nodes was fundamentally different. In the former, with a large infecting dose, *viz.* 10,900 organisms per 10 mg. of inoculum, the draining lymph nodes were already invaded within 24 hours, at a time when the draining lymph nodes in the normal animal were sterile. In the guinea pig on the other hand, even with larger numbers of bacilli of reinfection, the draining lymph nodes of the sensitized animal were sterile, although in the normal animal these were already invaded. A similar difference was noted in the spread of trypan blue in reinfected rabbits

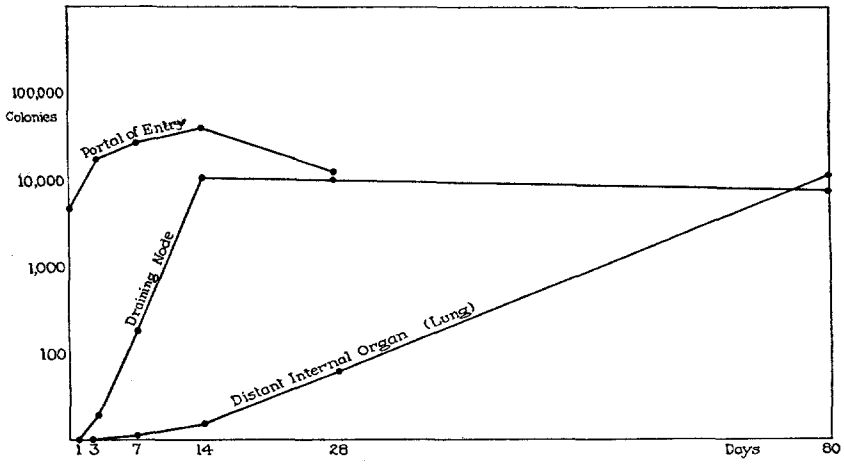
and guinea pigs. In the reinfected rabbit the draining lymph nodes contained more trypan blue than those of the normal rabbit. In the guinea pig, however, less trypan blue reached the draining lymph of the vaccinated than of the normal animal. Similar observations were made repeatedly in guinea pigs, both with trypan blue and bacilli, even with still larger reinfecting doses and without agar in the inoculum. The bacilli that rapidly invaded the draining lymph nodes and internal organs in the normal guinea pig underwent massive multiplication in the lymph nodes and liver for the first 2 weeks. In the immune animal on the other hand, few or none were found in the lymph nodes and internal organs 2 weeks after reinfection. In the 4th week, they accumulated but slowly in these draining lymph nodes at a time when large though reduced numbers persisted in the draining lymph nodes, and when uninterrupted multiplication continued in the liver of the normal guinea pig. A similar suppression of multiplication of the bacilli of reinfection that had invaded the draining lymph nodes and internal organs of immunized rabbits has been previously described.

Rôle of Local Immunity

Text-fig. 1 depicts graphically the fate of the bacilli in normal guinea pigs at the portal of entry, in the nearest draining inguinal node, in the more remote draining iliac node, and in a distant internal organ, the liver. The number of tubercle bacilli cultured from the agar focus on the 8th day is indicated but is not integrated in the graph, for this result is obviously atypical, as was evident both from subsequent developments in the agar focus, and also from many other such determinations, both in rabbits and guinea pigs. As noted above multiplication usually continues in the agar focus during the first 2 weeks after inoculation. It is evident that in all these four sites multiplication continues for the first 2 weeks. In the 4th week there is a marked reduction in the number of bacilli at the portal of entry, a much smaller but still definite reduction in the nearest lymph node, a complete cessation of accumulation but no significant diminution in the lymph node more removed from the agar focus and a practically uninterrupted and marked increase of the microorganisms in the distant liver. Essentially similar observations were



TEXT-FIG. 1. The fate of tubercle bacilli at the portal of entry and at sites more and more remote therefrom in normal guinea pigs.



TEXT-FIG. 2. The fate of tubercle bacilli at the portal of entry and at sites more and more remote therefrom in normal rabbits.

made in the rabbit (see Table I in the paper cited (4)), and are illustrated in Text-fig. 2.

It is evident therefore that the fate of the bacilli of primary infection and reinfection at the portal of entry and in the metastatic foci is, with one significant exception considered below, essentially the same in guinea pigs and rabbits. This behavior of the bacilli has already been described for the rabbit in the previous study. Here special attention is directed to the character of the developing immunity in the guinea pig and rabbit primarily infected. It was found that immunity, as determined by the fate of bacilli, appears most rapidly and intensely at the portal of entry of the microorganism, is progressively less effective in the lymph nodes more and more removed from this focus and, at the same time, is altogether ineffective in distant internal organs.

An important difference in the behavior of the two species is observed in the early dissemination of tubercle bacilli and trypan blue from the site of inoculation. With large doses, the bacilli of reinfection and trypan blue reach the draining lymph nodes of the sensitized rabbit more rapidly than they invade the same structures in the normal animal. In the sensitized guinea pig however, even with larger doses, both the bacilli of reinfection and the trypan blue are retarded in their spread to the draining lymph nodes as compared to that in the normal guinea pig. In the succeeding paragraphs an attempt is made to correlate this different behavior of the bacilli in the two species with their respective tissue response.

The Response of the Host

One day after inoculation of agar and tubercle bacilli subcutaneously into normal and vaccinated guinea pigs there is a large accumulation of fluid in the tissues surrounding the agar focus. At the periphery the exudate, situated in proximity to the collagenous fibres, is coagulated, forming a dense fibrinous network (Fig. 3). The outpouring of fluid is much more intense in the vaccinated than in the normal animal. The fibrinous deposits are much more marked in the sensitized animal, extending into the aureolar zone. The lymphatics in the normal animal are patent. In the vaccinated animal they are thrombosed by a fine fibrinous network (Fig. 1). The accumulation of cells about the focus of the vaccinated animal is much greater than that about the normal animal. Many of the polymorphonuclears in the former are necrotic; they are intact in the normal animal. The mononuclears are present in considerable numbers in the

vaccinated; they are scant in the normal guinea pig. The cells lie in a matrix of fibrinous network. This network is more widespread and denser in the vaccinated than in the normal guinea pig. In the normal animal the agar mass itself is broken up into small particles in immediate proximity to the fluid exudate; in the vaccinated this disruption of the agar is much less pronounced. The reaction in the sensitized rabbit differs from that of the sensitized guinea pig in the following respects. The accumulation of fluid about the focus is less pronounced, the polymorphonuclears are less injured and the fibrinous deposit is less marked around the blood vessels. It is coarse, consisting of bulky threads with large spaces between them (Fig. 4), while the fibrinous deposits in the guinea pig, on the other hand, consist of fine fibrillar strands lying close together (Fig. 3). The surrounding lymph vessels in the rabbit are open (Fig. 2), while in the guinea pig they are thrombosed (Fig. 1). The agar mass itself is much more broken up in the rabbit by bulky fibrinous strands than in the sensitized guinea pig.

There was no evidence of growth of the bacilli in any animal 1 day after inoculation. It is noteworthy, however, that while microscopically the bacilli were found in more prominent clumps in the vaccinated guinea pig the tissues of the latter yielded fewer colonies than the agar focus in the normal guinea pig.

In the draining superficial inguinal nodes of the normal guinea pig the marginal sinus was crowded with numerous granulocytes and they pervaded practically the entire node. Fluid distended the intermediate sinuses, and exfoliated macrophages with withdrawn processes were numerous. Many of them contained agar and trypan blue particles. In the draining superficial inguinal node of the vaccinated guinea pig, on the other hand, few granulocytes were present and little fluid was seen in the sinuses. The cells lining the sinuses were sessile, with their processes attached to the walls. They were not exfoliated. They contained little trypan blue, and no agar particles were identified in them. The reverse relationship held in the rabbit. The lymph node draining the agar focus in the normal rabbit was in all respects normal; both the marginal and intermediate sinuses were free of extraneous cells. In the sensitized rabbit on the other hand, the marginal sinus of the draining lymph node was distended with fluid and contained large numbers of polymorphonuclears. Macrophages with engulfed agar particles were seen lying free in the sinuses.

For the first 2 weeks following infection the bacilli in the acellular agar islands of the normal guinea pig were numerous. They appeared as loose masses of deeply acid-fast rods, from the periphery of which numerous bacilli radiated in all directions. They swarmed as dispersed forms uniformly deeply stained, with bulbous ends; branching forms were also seen. They were particularly numerous in proximity to the agar capsule and gradually diminished deep in the agar. They were more prominent in proximity to that portion of the capsule contiguous to the abdominal muscles than to that adjoining the skin. Occasionally deep in the agar were seen colonies which consisted of rounded or oval masses of extremely fine non-acid-fast granules, often arranged about a centrally situated unstained

spherule; from the periphery of this granular body extremely thin, long and short faintly acid-fast rods radiated.

In the immune animal, on the other hand, the bacilli were scanty throughout in the acellular agar, even in immediate proximity to the capsule. They appeared as minute dense clumps of acid-fast, extremely short rods, the individuals of which could not be easily discerned. There were none or few radiating forms about them. The clumps were spotted by numerous black or blue granules. Individual, very short, unevenly stained rods with polar bodies were seen. At times ill defined, weakly acid-fast globules were noted, in the center of which acid-fast granules were found. Rarely minute colonies were encountered, consisting of centrally situated non-acid-fast granules, from the periphery of which a few acid-fast rods sprouted.

In the normal animal the cellular reaction was diffuse, consisting of a large admixture of mononuclears, granulocytes and fibroblasts. No phagocytosis of tubercle bacilli by the mononuclears was noted on the 4th day after inoculation. Later these cells contained large numbers of long, deeply acid-fast rods with bulbous ends, often in the form of packets of parallel rods within the cell.

In the immune animal phagocytosis of tubercle bacilli by mononuclears was noted on the 4th day after reinfection. Well defined tubercles with mature epithelioid cells with well differentiated cytoplasm appeared on the 8th day. Minute foci of caseation were already present. The bacilli were scanty everywhere. They were short and beaded and were found especially in the caseous foci. Both in the normal and in the immune animal the cells lay in a matrix of fibrin. This was particularly conspicuous and extensive in the immunized animal, the cells often lying as if in a basket of intercellular fibrillar substance. By the 2nd week bacilli swarmed within the cells of the normal animal. They were particularly numerous in those central foci of caseation still infiltrated with large numbers of polymorphonuclears. Polymorphonuclears, however, were not limited to these foci but persisted practically throughout the whole lesion. The mononuclears had not yet assumed the shape of mature epithelioid cells; there was as yet no differentiation of the cytoplasm; their processes were still stretched in different directions. The lesion was diffuse and not nodular, invading the surrounding muscle bundles. The latter were often necrotic, apparently choked by the advancing tuberculous process. The enmeshed blood vessels in the necrotic zone were partially thrombosed. Underneath the skin the capsule was less specific in character; fibrous tissue was prominent here.

In the necrotic zone abutting against the agar in the immune animal all the vessels were completely thrombosed. Some of the tubercles were already undergoing regressive changes. The epithelioid cells were rounded and without processes; there was almost no accompaniment by polymorphonuclears. Tubercle bacilli were everywhere scant; short beaded forms were found. The non-specific fibrous tissue formation was much more extensive in the immune animal, and under the skin it was present almost to the exclusion of any specific changes.

In the 4th week the capsule of the normal guinea pig had undergone massive

and diffuse caseation. In those caseous foci where only nuclear debris remained, the bacilli were few, beaded, and short. Caseous foci in an earlier state of development still contained numerous long deeply staining acid-fast organisms. In the vaccinated guinea pig, on the 4th week the same difference from the normal obtained as noted in the 2nd week. Caseation was much less in evidence and fibrous tissue deposits in the capsule were conspicuous.

Thus it is clearly seen that the essential features of the immune response in the rabbit and guinea pig are the same. In both there is suppression of growth of the bacilli of reinfection, both extra- and intracellularly. In both there is a more rapid mobilization of the mononuclears. In both the polymorphonuclears soon disappear from the site of reinfection. In both the phagocytic properties of the mononuclears are greater in the immune than in the normal animal. In both the destruction of the bacilli in the immune animal is associated with a more rapid nodular formation of mature epithelioid tubercles. It is true, however, that nodular tubercle formation is more pronounced in the immune rabbit than in the immune guinea pig; the reaction, even in an immune guinea pig, is still to some extent diffuse.

The chief differences in the response of the two species to reinfection are two. One is the degree of injury the bacilli exert on the tissues of the sensitized animal of these two species. The other is the response of the intercellular substance.

The more highly sensitized tissues of the guinea pig respond with a greater outpouring of fluid, presumably resulting from a greater injury to the vessel walls. The focus becomes shunted off by the clotting of the exudate and the thrombosis of the lymphatics (Fig. 1). The clot is dense and intimate in the guinea pig (Fig. 3); it is loose and coarse in the rabbit (Fig. 4). It is obvious that these extracellular factors form a more effective barrier against the easy dissemination of particulate matter from the site of reinfection in the guinea pig than in the rabbit, where the fibrinous deposits are less pronounced, more porous, and where the draining lymphatics remain open (Fig. 2). It is noteworthy that not only are tubercle bacilli and trypan blue retarded in their passage from the site of reinfection, but agar particles are also prevented from reaching the draining lymph nodes of the vaccinated guinea pig. In the latter they were never found. In

the rabbit, however, the agar is carried to draining lymph nodes of both normal and immunized rabbits. See Fig. 10 and 11 in the paper cited above (4).

The amount and character of the intercellular substance in the two species appear to be operative in the same direction. The fibrinous network in which the cells are lodged is dense and intimate in the guinea pig and very conspicuous (Fig. 7). It is coarse and loose and of much smaller extent (Fig. 8) in the rabbit. Fibrous tissue formation is very much greater in the guinea pig than in the rabbit.

It is interesting to note in this connection that clotted plasma of normal rabbits differs from clotted plasma of guinea pigs. The individual threads in the clot of a rabbit are coarse, with large spaces between them, forming a large meshed sieve (Fig. 6). The individual threads of the clot of guinea pigs are very fine and closely applied, with minute spaces between them, forming a fine sieve (Fig. 5). Furthermore, the plasma clot of a rabbit is soft and friable, and on centrifugation yields its enmeshed serum with ease. The plasma clot of a guinea pig on the other hand is much firmer and less fragile, and on centrifugation its enmeshed serum separates with greater difficulty.

Further observations that suggest mechanical differences in the character of the respective inflammations in the two species have been made. Normal and tuberculous rabbits and guinea pigs were immunized with (*a*) formalinized typhoid bacilli, (*b*) a tuberculoprotein, TPT, (8) and (*c*) horse serum. When the antibody titre of the serum of these animals had attained a certain level, they were given an intrapleural injection of aleuronat-starch. 1 or 2 days later the resulting exudate, freed from cells, was titrated for its agglutinins or precipitins. At the same time the concentration of these antibodies in the corresponding sera of these animals was determined. The results are recorded in Table II.

It is seen that the concentration of agglutinins in the exudate of both normal and tuberculous rabbits is the same as that of the corresponding sera. In the tuberculous guinea pigs, however, while the antibody content of the exudate is also the same as that of the serum of the same animal, it is relatively higher than that of the exudate in a normal guinea pig, the antibody titre of which is lower

than that of its corresponding serum. Again in tuberculous rabbits the precipitin titre of the exudate is often lower than that of the synchronous concentration in their corresponding sera. In tuberculous guinea pigs, however, these antibodies accumulate in much higher titre in the exudate than in the circulating blood.

In other words not only do more precipitins pass from the blood

TABLE II

Synchronous Concentration of Antibodies

In the Blood Serum and in Inflammatory Exudates of Normal and Tuberculous Rabbits and Guinea Pigs

Rabbits			Guinea pigs			Rabbits			Guinea pigs		
Agglutinins			Agglutinins			Precipitins*			Precipitins†		
Rabbit No., normal or tuberculous	Titre of serum	Titre of exudate	Guinea pig No., normal or tuberculous	Titre of serum	Titre of exudate	Rabbit No., normal or tuberculous	Titre of serum	Titre of exudate	Guinea pig No., normal or tuberculous	Titre of serum	Titre of exudate
									15-8 Normal	320	160
50-1 Normal	1280	1280	66 Normal	500	125	G2-20 Normal	5000	5000	15-9 "	640	320
E 3-9 "	1280	1280	69 "	1000	500	B3-12 "	5000	500	16-4 "	320	640
H 2-3 "	320	320	68 "	500	250	C2-19 "	50,000	5000	16-1 "	160	640
E 3-10 "	320	320	70 Tbc.	500	500	A3-3 Tbc.	60,000	80,000	16-3 "	320	640
2 Tbc.	5120	1280	71 "	250	250	C3-1 "	100,000	70,000	14 Tbc.	160	1280
7 "	1280	1280	36 "	500	500	C3-6 "	90,000	80,000	16 "	320	640
A 3-6 Tbc.	1280	1280							40 "	640	2560

* *Versus* tuberculo-protein.

† *Versus* horse serum.

into the exudate to a tuberculous guinea pig than into the site of inflammation of a tuberculous rabbit, but after they have permeated the vessels of the guinea pig they tend to accumulate there until they attain a higher concentration than that in their corresponding serum. This would suggest the following mechanism. The vessels of a tuberculous guinea pig are more injured by the irritant than the vessels of a tuberculous rabbit; hence more antibodies will exude from the blood of the former. But this will not account for the higher concentration of antibodies in the exudate. It may be, however, that the efferent lymphatics at the site of a local non-specific inflammation

in a tuberculous guinea pig are plugged, just as occurs in response to a specific irritant, whereas these vessels in a tuberculous rabbit remain open. In this case the antibodies will leave the site of inflammation in the rabbit, but will be blocked in the guinea pig.

Rôle of Extracellular Factors in the Inhibition of Growth of Tubercle Bacilli of Reinfection

It was shown in the previous study (4) with the agar focus in rabbits and confirmed in the present investigation in guinea pigs that in the acellular agar islands of the normal animal, into which the body fluids penetrate, the bacilli multiply unhindered, whereas in the immune animal a marked inhibition of their growth was evident in these cell-free areas saturated with the immune body fluids. While the observations are suggestive, their interpretation is open to question. The growth of the bacilli in the agar focus is marked at the periphery in proximity to the cellular infiltration. Deeper within the agar the growth is scanty, even in the normal rabbit or guinea pig. Now it has been noted that in the normal animal of both species the agar is broken up into small particles by the invading exudate. In the immune animal on the other hand these particles are larger. It is conceivable, therefore, that the observed suppression of growth of the bacilli in the immune animal may be due, not to the bacteriostatic properties of its body fluids, but to the less effective penetration of these, due to the fact that in the tuberculous animal the agar is little dispersed and in large aggregates. To answer further questions, it was desirable to set up an experiment in which the cells are entirely and permanently kept out from the site of *in vivo* multiplication of the bacilli, although exposed to body fluids.

After numerous trials the following procedure was adopted. Pure, undyed silk, the threads of which were so woven that the interstices between the fibres formed parallelograms about 42 micra in length and 18 micra in width, was sewn into bags. Into the mouth of these bags the lipped rim of a pyrex glass cannula was fastened; the cannula was constricted at its distal third. This bag with its attached cannula was autoclaved and dried under sterile conditions. Because of the shape of the bag, Elford's technique for the preparation of graded collodion membranes of known porosity was inapplicable (9). They were therefore coated with an arbitrary concentration of Mallinckrodt's parlodion which had been autoclaved and dried with sterile precautions. The concentration of parlodion

varied between 2 and 3 per cent by weight. The solvent was 75 to 80 per cent absolute alcohol and 20 to 25 per cent absolute ether. The dry sterilized bags with their attached cannulas were immersed for several hours to several days in the collodion solvent to expel all air bubbles. They were then submerged in the parlodion solution for a similar period. With painstaking sterile precautions, the bags were removed from the parlodion solution, which was allowed to drain off completely. These were now dried in air for about 6 minutes in a vertical position, as routine. The emersion was repeated several times. The junction between the glass cannula and the silk was covered with a thick layer of collodion. Bags so impregnated proved entirely impervious to cells (Figs. 9, 10, 11 and 12) and readily permeable to body fluids. 3 to 4 per cent of molten agar in saline cooled to 50°C. was mixed with a suspension of virulent bovine tubercle bacilli in trypan blue or India ink. By means of a syringe with a long needle the bags were filled with this mixture up to the level of the glass cannula. The cannula was then sealed in the flame at the point of constriction and, after cooling, was placed in the peritoneal cavity of a normal or tuberculous rabbit. At the same time a weighed portion of the mixture of tubercle bacilli and agar was reserved for culture to determine the number of bacilli present in the inoculum before it was placed in the animal.

In a given experiment the preparation of the bags to be used for a normal and tuberculous animal was as nearly identical in each individual procedure as was possible. In some instances, another bag, prepared at the same time, was filled with sterile salt solution, sealed in the flame and placed in the peritoneal cavity of the normal and the tuberculous rabbit simultaneously with the introduction of the bags containing agar and living tubercle bacilli. At different intervals of time, but most often after 2 weeks sojourn in the peritoneal cavity of the normal and immunized animals, *i.e.*, the time of maximum multiplication of locally injected tubercle bacilli, the bags were removed and opened. A large sample of the solid, unbroken, transparent agar was removed, weighed, and used for culture to determine the fate of the bacilli in the agar within the bags. The portion of agar immediately adjoining this sample, together with the tissue membrane that had formed outside and about the collodion-impregnated silk bag in its sojourn in the peritoneal cavity, was prepared for histological study. In some cases, immediately upon opening the silk bag, portions of the agar within were covered with mineral oil for determination of its pH concentration by the method of Hastings and Sendroy (10). On the occasions when blank salt solution-containing bags were also present in the peritoneal cavity, the protein concentration of the fluid within them was determined by the gravimetric method as given by Peters and Van Slyke (11). At the same time its pH concentration was determined. Table III presents the results obtained in 9 such experiments.

It is clearly seen that in all but a single instance the growth of the bacilli within the bags situated in the peritoneal cavity of normal animals is far greater than that taking place within bags placed in a

tuberculous animal. This is sharply brought out in the column listing the ratio between the number of colonies cultured from the agar after its sojourn in the peritoneal cavity and the number obtained from the original inoculum. It will be noted that the number of bacilli within the bags placed in normal rabbits varied from 1.7 to

TABLE III

Fate of Virulent Bovine Tubercle Bacilli within Collodion-Impregnated Silk Bags Placed in the Peritoneal Cavity of Normal and Tuberculous Rabbits

Length of stay of bags in the peritoneal cavity	Number of colonies in inoculum	Number of colonies in bags of				Ratio between number of colonies in bag and in inoculum of		pH of agar in bags of	
		Normal rabbits		Tuberculous rabbits					
		Rabbit No.	Colonies in bag	Rabbit No.	Colonies in bag	Normal rabbits	Tuberculous rabbits	Normal rabbits	Tuberculous rabbits
days									
13	11,700	1	50,000	A 31-6	13,200	4.3	1.1		
14	5900	30-4	140,000	39-6	0*	23.7	—		
14	4900	4	1,400,000†	31-0	33,000†	285.7	6.7		
14	4900	4	190,000‡	31-0	12,000‡	38.7	2.5		
4	4300	3	2600	31-1	300	0.6	0.07		
5	13,200§ 23,200‖	10	210,000	83	30,000	15.9	1.3	7.40	7.40
14	8350	5	160,000‡	E 3-9	1000‡	19.1	0.1	7.25‡	6.95‡
14	8350	5	33,000†	E 3-9	1000†	4.0	0.1	7.35†	7.05†
13	3100	6	133,000	E 3-10	156,000	42.9	50.3	7.35	7.30
14	1500	17-8	2600	6	300	1.7	0.2	7.28	7.05
14	75,000§ 104,000‖	20-3	2,700,000	FM 2	230,000	36.0	2.2	7.22	7.31

* After treatment with sulfuric acid.

† Bag of about 12 mm. diameter.

‡ Bag of about 5 mm. in diameter.

§ Inoculum placed in bag of normal rabbit.

‖ Inoculum placed in bag of tuberculous animal.

285.7 times the number present in the original inoculum. Within the bags placed in tuberculous animals on the other hand, the increase, with the exception of that of E 3-10 noted above, ranged between 1.1 to a maximum of 6.7 that of the original inoculum. In only one instance of a bag placed in a normal rabbit was there an actual re-

duction in the number cultured, as compared with that of the inoculum. It is interesting that in this case the reduction of the number of bacilli within a similar bag placed simultaneously in a tuberculous animal was ten times greater, and that three additional instances of such reduction were found within bags placed in tuberculous animals. The range of dosage in the inoculum varied greatly in different experiments. Presumably also the thickness of the collodion differed in different sets, although in a given set there was little difference in the thickness of the collodion membrane of the bags placed simultaneously in a given pair of normal and tuberculous animals.

Frequently both in normal and tuberculous rabbits, within the glass cannula attached to the bags, above the level of the agar, a clear, cell-free, protein-containing fluid collected, which did not coagulate after several days at 37°C. Likewise within the bags containing salt solution only, identically prepared and placed simultaneously within the peritoneal cavity of normal and tuberculous rabbits, a similar fluid, containing about 3 per cent protein, which failed to clot, was present. It is plain, therefore, that the body fluid penetrated the collodion-impregnated bags placed both in normal and tuberculous animals, but this fluid in a tuberculous rabbit is definitely bacteriostatic as compared with that penetrating a membrane of similar character in a normal animal.

As can be seen from columns 9 and 10 in Table III the pH of the agar within bags in tuberculous animals is frequently considerably lower than that in bags in normal rabbits. However, in three separate experiments where blank, saline-containing bags were examined, there was no difference in the pH of the fluid in these bags placed in a normal or tuberculous animal. Whether this increased acidity of the agar in these instances is due to the differing behavior of the bacilli within them or to some other cause cannot be stated.

Histological preparations of these bags revealed that the contents of the bags remained absolutely cell-free even after 14 or more days sojourn in the peritoneal cavity (Figs. 13, 15 and 16). There was no fibrinous deposit within the bags. Presumably fibrinogen failed to penetrate the collodion, as the protein-containing fluids that penetrated these sacs failed to clot. As a result, the agar remained as

one solid mass, unbroken by the fluids that seeped into it. It is seen therefore that the objections that could have been raised to the interpretation of the results of the agar focus technique as stated above are met in this procedure. As with the subcutaneous agar focus the growth within the bag is chiefly peripheral, being densest in a narrow zone close to the collodion membrane and rapidly decreasing in the deeper layers (Fig. 13). A similar distribution of the bacilli is seen in older caseous foci of infected tissues as illustrated in a caseous lymph node in Fig. 14.

There is usually considerable correspondence between the number of bacilli cultured from within the bags and their histological appearance. They are numerous and appear as actively growing colonies in the bags situated in normal animals (Fig. 9). They are scanty, poorly growing, and often degenerated within bags that had sojourned within the peritoneal cavities of the tuberculous animals (Fig. 10). The differences between them are essentially those previously described in the subcutaneous agar focus of normal and immunized rabbits and guinea pigs.

Two points however require emphasis. It is clear in this procedure that the bacilli frequently grow *in vivo* by the subdivision of the original clump into extremely fine, barely visible, non-acid-fast granules and thin short rods from the periphery of which non-acid-fast, and acid-fast bacilli bud out in all directions (Figs. 15 and 16). Similar observations have been made, as noted above, in the subcutaneous agar focus.

The tissue membrane that forms about these bags is frequently thicker in the normal (Fig. 11) than in the tuberculous animal (Fig. 12). This is to be associated with the fact that the bacilli gradually grow through the collodion which is always saturated with the body fluids. Since the growth is much more pronounced within the bags in normal animals than in those situated in tuberculous animals, this penetration of the bacilli through the surrounding collodion is much more frequent and extensive about bags in normal animals, and tuberculous changes in the investing membrane surrounding these sacs are necessarily much more frequent and conspicuous than in the tissue membrane investing the collodion-impregnated silk bags remaining in the peritoneal cavities of tuberculous rabbits.

SUMMARY AND DISCUSSION

A comparative analysis of the behavior of bacilli of reinfection in immunized and sensitized rabbits and guinea pigs, together with a consideration of the associated host responses in the two species, reveals that the fate of the bacilli and the immune processes of the host are essentially similar in both. However, the bacilli of reinfection are more effectively fixed at the portal of entry in a sensitized and immunized guinea pig than in a comparable rabbit. This has been correlated with the degree of allergy developed by these two types of animals. It is well known that the tissues of a guinea pig become more highly sensitized to the tubercle bacillus than those of a rabbit. The contact of the microorganism with the tissues of the former exerts far more injury upon them than upon those of the latter. A more abundant exudate forms in the guinea pig, the exuded plasma coagulates and, most significantly, thrombosis of the adjoining lymph vessels (Fig. 1) quickly shunts off the focus of reinfection. It is possible that the thrombokinase released by the injured cells may play a part in the observed phenomena. Experiments are under way testing this conception. In the rabbit, on the other hand, the injury of the tissues by the bacilli of reinfection is much less pronounced, coagulation of the exuded plasma is less conspicuous and, particularly, the adjoining lymph vessels remain open (Fig. 2). It would seem also that the fine sieve formation of the clot in the guinea pig (Figs. 3 and 5) as compared with the coarse sieve arrangement of the fibrinous network of the rabbit (Figs. 4 and 6) would also aid in the fixation of substances at the site of inflammation in the former. It is interesting in this connection that the site of a tuberculin reaction in a guinea pig is firm and indurated, whereas in a rabbit it is soft and boggy.

That mechanical differences in the character of the inflammation of reinfection in the two species rather than specific immune processes are involved in the more effective fixation of the bacilli of reinfection in the guinea pig, is suggested by the fact that entirely unrelated substances, such as trypan blue and agar particles, are more effectively localized at the site of reinfection in the guinea pig than in the rabbit. Furthermore in tuberculous guinea pigs at the site of a

non-specific inflammation, blood precipitins accumulate in much higher titre than at a similar site in a tuberculous rabbit. The precipitin titre of the exudate in a tuberculous guinea pig is several times that of the simultaneous titre of its serum. In a tuberculous rabbit it is often lower than the serum titre. It is obvious that only non-specific characteristics of the inflammation in the two species can be involved. It is clear, therefore, that allergic inflammation in the highly sensitized guinea pig mechanically hinders the spread of tubercle bacilli from the site of reinfection. Since the degree of sensitivity of the infected human being is very much higher than that of the guinea pig, it is logical to expect that the inflammation of reinfection in man will have even greater fixing capacities than that of a guinea pig. However the character of the fibrinous network in man is more like that of a rabbit. The plasma clot of man (Fig. 17) forms a network of coarse fibres with large spaces between them. Therefore the exact position of man in this relation still remains uncertain.

While Rothschild (1) and associates demonstrated that desensitization with tuberculin does not lessen the immunity of vaccinated guinea pigs for a considerable time after reinfection, Willis and Woodruff (12) have found that if the desensitized animals are permitted to die from their reinfection they survive a shorter period, develop more extensive pulmonary disease and harbor larger numbers of viable tubercle bacilli in their internal organs than allergic animals similarly reinfected but not desensitized. It is difficult to state to what extent the results of this treatment with tuberculin are due to the removal of the exaggerated inflammatory responsiveness of the tissues to the tubercle bacillus. It is clear, however, that the administration of this agent interferes to some extent, at least, with the immune process.

The importance of the early dissemination of baccilli from the portal of entry is emphasized by the study of the behavior of the bacilli at this site. It has been shown that both in rabbits and in guinea pigs the bacilli are being effectively destroyed at the portal of entry, at a time when they are multiplying practically without interference in distant internal organs (Text-figs. 1 and 2). It is the extension of the tuberculous process in the metastatic foci, and

not at the site of entry of the microorganism, that is significant for the fate of the animal.

Whether this progressive diminution in the inhibition of the multiplication of the bacilli in sites more and more remote from the portal of entry is an expression of a graded local immunity, which develops most rapidly and intensely at the portal of entry and appears later in more distant foci until, at last, the whole organism is immunized, is not so clearly established from these data. It has been shown in previous studies that the maximum immunity developed by a given organ, such as the lung, is never as effective as that of the liver. Nevertheless it is suggestive that Stewart (13) has shown that tuberculin sensitivity develops earlier and in greater intensity in proximity to the primary tuberculous lesion than at a distance from it. Again, as has been shown in a posthumous publication of Sewall (14), the secondary nodule which results 29 days after reinfection of the skin close to the cutaneous primary lesions is much smaller than that which results from reinfection of a skin site remote from the primary lesion. If the size of the lesions is taken as a measure of immunity, it is plain that the immunity in close proximity to a primary focus is greater than that at a more remote site in the same tissue, even in animals that had harbored tuberculosis for 90 days previous to the reinfection; for these were the conditions of the experiments cited. It is significant in this connection that McMaster and Hudack (15) have demonstrated that antibodies are at first present in higher concentration in the lymph nodes draining the site of introduction of the antigen than in the general circulation.

Numerous attempts have been made to demonstrate *in vitro* bacteriocidal properties of serum derived from animals immunized to tuberculosis, but without success. To cite but one experiment, Römer and Joseph (16) exposed 0.000,000,1 mg. of tubercle bacilli, which was the minimal dose still capable of producing tuberculosis in a guinea pig, to the action of 2 cc. of immune serum of a highly sensitized sheep for 24 hours without reducing their virulence. It has been shown in this study that tubercle bacilli exposed *in vivo* to the body fluids of tuberculous animals within collodion-impregnated silk bags, in the complete absence of cells (Figs. 11 and 13) or any other known bacteriostatic factor, are markedly inhibited

in their growth (Fig. 10) for much more extensive multiplication takes place when tubercle bacilli are exposed under identical conditions to the body fluids of a normal animal (Fig. 9). The gradual disappearance of tubercle bacilli from acellular caseous foci is a parallel observation. On the other hand the inordinate growth and swarming of tubercle bacilli in old cell-free caseous foci undergoing softening indicates that other unknown factors may intervene to overcome this bacteriostatic property of the body fluids in the immune animal. To what extent, if any, the local accumulation of antibodies from the blood at the site of reinfection in highly sensitized animals aids in their local suppression of growth is uncertain.

The complete absence of cells from these *in vivo* growth sites of the tubercle bacillus has afforded an opportunity to confirm the studies of Kahn (17). Under certain conditions, the tubercle bacillus does not grow by fission only, but by preliminary subdivision into fine non-acid-fast granules from which both non-acid-fast and acid-fast bacilli sprout (Figs. 15 and 16). It is perhaps this non-acid-fast state of the bacilli which may explain the incongruity often observed between cultural and inoculation methods on the one hand, and histological procedures on the other, in the demonstration of tubercle bacilli.

CONCLUSIONS

1. The fate of bacilli of reinfection at the portal of entry and in metastatic foci, and also the associated host responses, are essentially similar in rabbits and guinea pigs.

2. However, in the guinea pig tubercle bacilli of reinfection are more effectively fixed at the portal of entry than in the rabbit.

3. The guinea pig fixes at the site of reinfection unrelated substances, such as trypan blue and agar particles, more effectively than the rabbit.

4. At the site of a local non-specific inflammation precipitins from the circulating blood accumulate in higher concentration in tuberculous guinea pigs than in tuberculous rabbits.

5. These differing fixing capacities of the two species are associated with differences of extracellular character in the inflammation resulting from reinfection. (*a*) In the guinea pig, whose tissues are

highly sensitized and greatly injured by the tubercle bacillus, the lymphatics adjoining the site of reinfection become thrombosed. In the rabbit whose tissues are moderately sensitized and less injured by the tubercle bacillus the corresponding lymphatics remain open. (*b*) In the guinea pig the fibrinous network at the site of inflammation forms a fine sieve-like structure. In the rabbit this network forms a coarse sieve-like barrier.

6. In rabbits and guinea pigs primarily infected, the destruction of tubercle bacilli takes place first and most extensively at the portal of entry. At this time they are less effectively destroyed in the nearest metastatic foci. Simultaneously they are still growing without hinderance in such foci in remote internal organs.

7. The cell-free body fluids of normal animals support the growth of tubercle bacilli *in vivo*. The body fluids of tuberculous animals under the same conditions are bacteriostatic for this microorganism.

8. Tubercle bacilli often multiply by preliminary subdivision into non-acid-fast granules, from which the acid-fast rods sprout. This confirms the work of Kahn.

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EXPLANATION OF PLATES

All sections were prepared from tissues stained with Masson's trichrome procedure except those depicted in Figs. 9, 10, 13, 14, 15 and 16 which were stained by the Ziehl-Neelsen method, and counterstained with hematoxylin. The magnifications given are approximate.

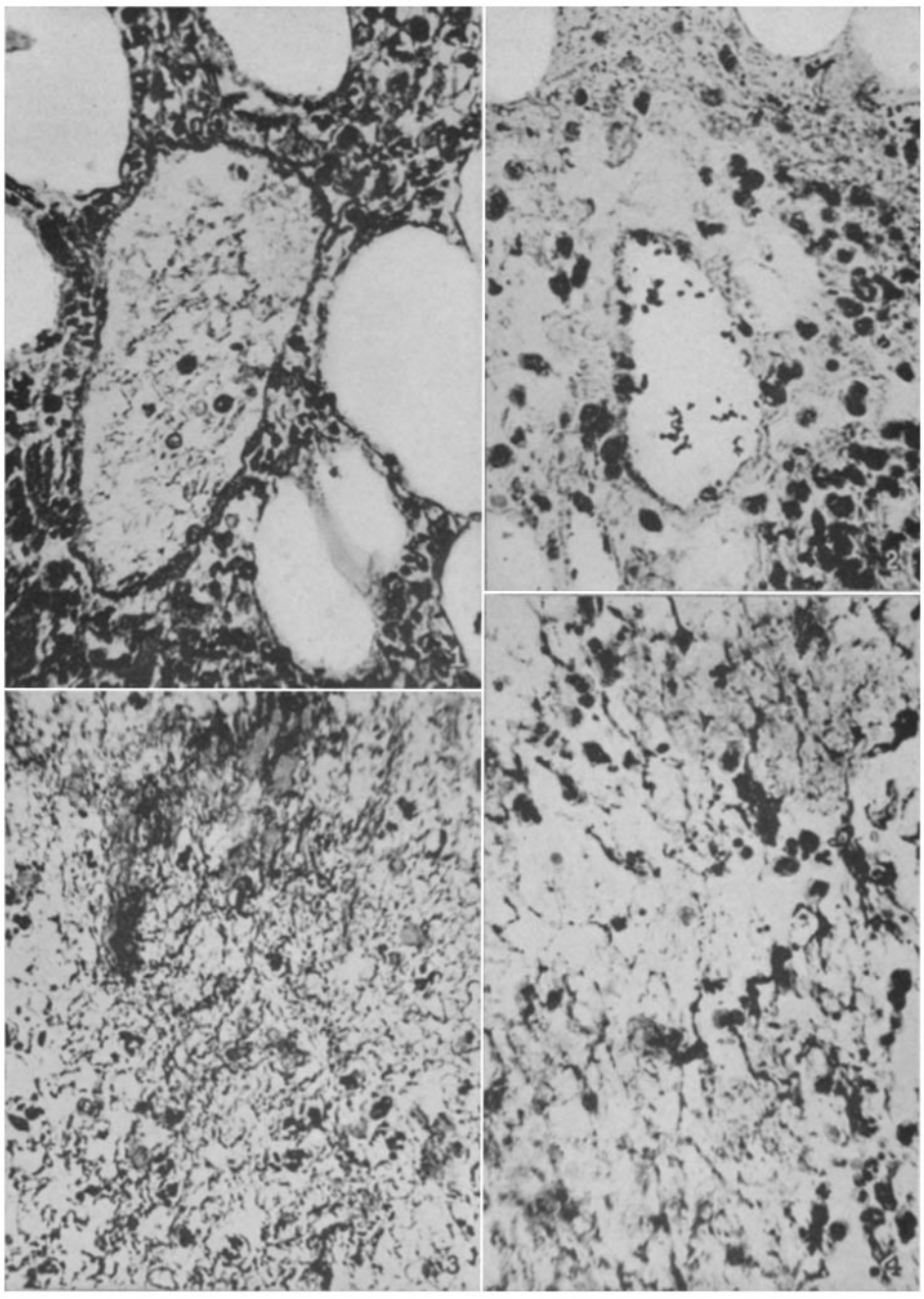
PLATE 32

FIG. 1. Thrombosed lymphatic at the site of inoculation of a BCG vaccinated guinea pig, 1 day after reinfection. The lymph node draining this focus was sterile. × 700.

FIG. 2. Open lymphatic at the site of inoculation of a tuberculous rabbit, 1 day after reinfection. A few coarse fibrin threads are present within the lumen. The lymph node draining this focus yielded 16 colonies. × 700.

FIG. 3. The fibrinous exudate at the site of reinfection in the sensitized guinea pig shown in Fig. 1. The fibrin threads are fine and the spaces between them are small, forming a dense sieve-like structure. × 700.

FIG. 4. The fibrinous exudate at the site of reinfection in the sensitized rabbit shown in Fig. 2. The fibrin threads are bulky and the spaces between them are large, forming a coarse sieve-like structure. × 700.



(Lurie: Mechanism of immunity in tuberculosis)

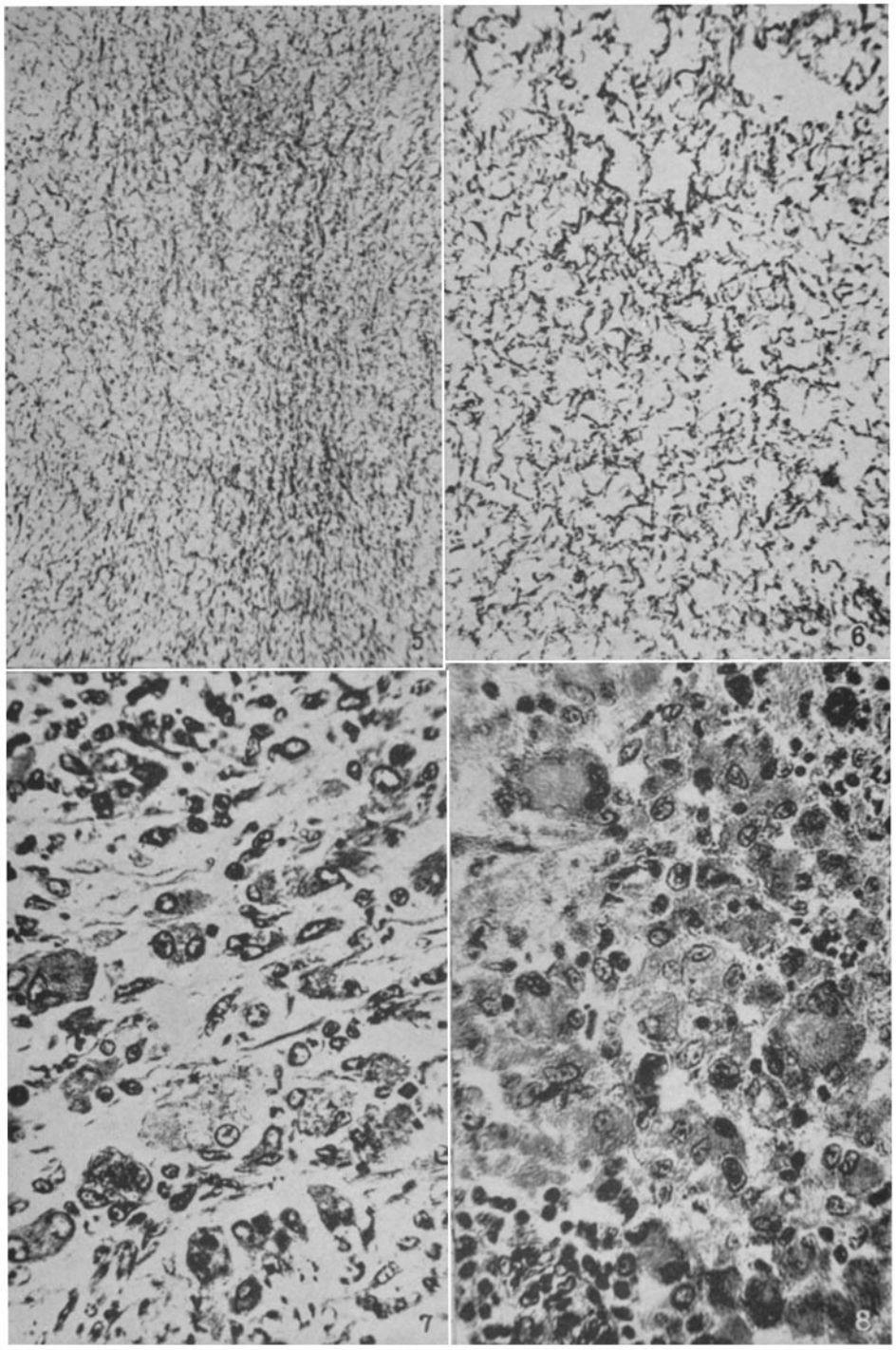
PLATE 33

FIG. 5. Clotted plasma of a normal guinea pig. The closely lying fine fibrin threads form a dense network. × 700.

FIG. 6. Clotted plasma of a normal rabbit. The bulky dispersed fibrin threads form a coarse network. × 700.

FIG. 7. The capsule surrounding the agar focus in vaccinated guinea pig 18-2, 2 weeks after reinfection. Fibrillar intercellular substance is conspicuous. × 700.

FIG. 8. The capsule surrounding the agar focus in tuberculous rabbit 14-3, 2 weeks after reinfection. Intercellular substance is very scant. × 700.



(Lurie: Mechanism of immunity in tuberculosis)

PLATE 34

FIG. 9. Tubercle bacilli growing in small bag placed in normal rabbit 5, after 2 weeks stay in the peritoneal cavity. 160,000 colonies were recovered from 10 mg. of agar within this bag. Ratio between number of colonies in bag and that in original inoculum, 19.1. × 700.

FIG. 10. Tubercle bacilli growing in small bag placed in tuberculous rabbit E 3-9 simultaneously with that placed in normal rabbit 5, shown in Fig. 9, after 2 weeks stay in the peritoneal cavity. 1000 colonies were recovered. Ratio between number of colonies in bag and that in original inoculum, 0.1. × 700.

FIG. 11. Thick membrane investing collodion-impregnated bag placed in normal rabbit 4, after 14 days stay in the peritoneal cavity. The leukocytes come up to the collodion but do not pass through. Ratio between number of colonies recovered from agar within this bag and that from original inoculum, 285.7. × 160.

FIG. 12. Thin membrane investing the collodion-impregnated bag placed in tuberculous rabbit 31-0 simultaneously with bag placed in normal rabbit 4 shown in Fig. 11, after 14 days stay in the peritoneal cavity. From above downwards the following structures are encountered: the tissue membrane investing the bag, the collodion on the outside of the silk threads, the silk threads cut across, the inner layer of collodion, and the acellular agar within the bag. Ratio between number of colonies recovered from agar within this bag and that from original inoculum, 6.7. × 160.

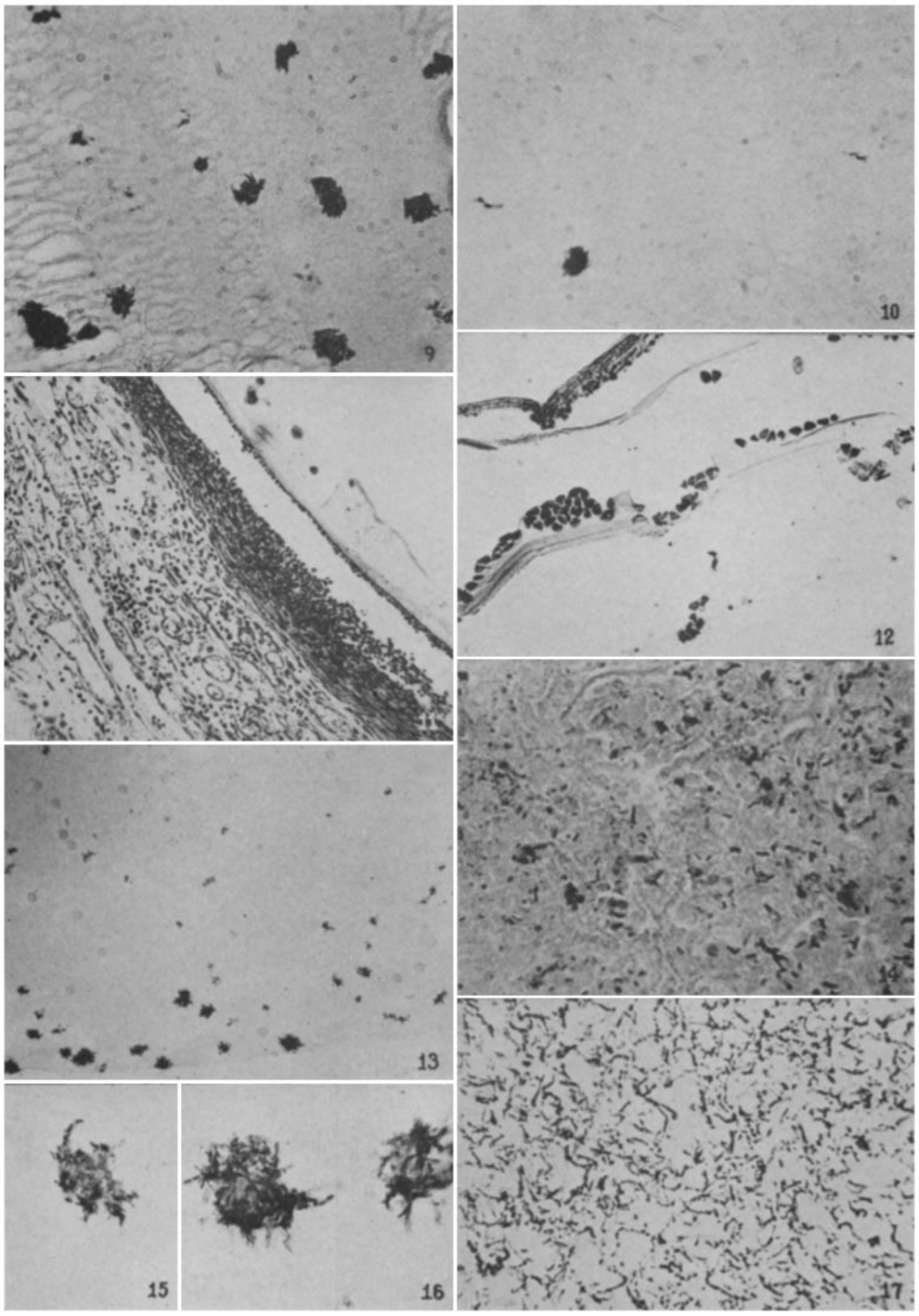
FIG. 13. Growth of tubercle bacilli within collodion-impregnated silk bag placed in normal guinea pig 15-2, after 4 weeks stay in the peritoneal cavity. Cells are completely absent. The colonies of tubercle bacilli are limited chiefly to a narrow zone on the periphery of the unbroken agar adjoining the sac, which was below the figure. The growth is scant in the deeper regions. × 160.

FIG. 14. The periphery of a caseous lymph node of a rabbit injected with bovine tubercle bacilli. The microorganisms are present in large numbers near the edge of the node close to the fibrous capsule, which is situated to the right of this figure. The bacilli are fewer in the deeper layers of the node. × 700.

FIG. 15. Colony of tubercle bacilli growing in bag placed in normal rabbit 4 after 14 days stay in the peritoneal cavity. The light particles in the center are non-acid-fast granules. The dark rods are short acid-fast bacilli sprouting from the periphery of the granular body. × 1500.

FIG. 16. Colonies of tubercle bacilli growing in same bag as in Fig. 15. Long, deeply acid-fast, dark rods are seen radiating from a centrally situated light, non-acid-fast, faintly granular body. × 1500.

FIG. 17. Clotted human plasma. The network formed by the fibrin resembles that of a rabbit shown in Fig. 6. × 1500.



(Lurie: Mechanism of immunity in tuberculosis)